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NOVEL EMBRYONIC CELL POPULATIONS AND METHODS
TO ISOLATE SUCH POPULATIONS

FIELD OF THE INVENTION

The present invention is related to novel populations of
5 embryonic cells and methods to produce such populations of
cells. More particularly, the present invention relates to
pluripotent progeny of embryonic stem cells and the use of
such populations to identify compounds expressed during the
differentiation of embryonic stem cells into a variety of cell
10 types.

BACKGROUND OF THE INVENTION

Multicellular animals are derived from a clone of cells
descended from a single original cell, the fertilized egg.
Embryogenesis involves the division and differentiation of
15 multi-potential cells, each cell having the ability to develop
into multiple cellular lineages. Phenotypically, the cells of
such lineages can vary substantially, such as blood cells,
muscle cells and neural cells, being specialized.

A wide spectrum of diseases may be treated based upon
20 both the possession of a population of cells having multi-
lineage potential and an understanding of the mechanisms that
regulate embryonic cell development. For example, the
capacity to generate a new population of hematopoietic cells
is the basis of bone marrow transplantation, which is
25 currently used as a treatment for a growing number of diseases
including anemia, leukemia and breast cancer. In addition,
transplantation of genetically altered multi-potential cells

has been considered as potential therapy for a variety of different diseases including AIDS.

One of the major barriers to both the treatment of diseases and the study of the process by which an undifferentiated embryonic cell becomes committed to a particular developmental pathway is the lack of access to populations of embryonic cells that are sufficiently multi-potent to be able to develop into various lineages. In particular, much attention has been paid to the use of bone marrow stem cells as a source of multi-potential cells for therapy and experimental use. Bone marrow stem cells, however, have limited use because such populations of cells comprise a subpopulation of complex hematopoietic tissue and, therefore are rare. In addition, bone marrow stem cells have not been grown as a substantially homogeneous population in tissue culture.

Following fertilization, an egg divides over a period of days to form a blastocyst. A blastocyst includes a hollow ball of cells having an inner cell mass and a fluid-filled cavity, both encapsulated by a layer of trophoblast cells. The blastocyst then implants into the uterine wall and enters into the embryonic stage of development characterized by the formation of the placenta, the development of major internal organs and the appearance of major external body structure.

Cells from the inner cell mass of an embryo (i.e., blastocyst) can be used to derive a cell line capable of being maintained in tissue culture that is referred to as embryonic stem (ES) cells. The use of ES cells to obtain hematopoietic populations of differentiated cells has been suggested in 5 Burkett et al., pp. 698-708, 1991, *New Biologist*, Vol. 3; Schmitt et al., pp. 728-740, 1991, *Genes and Development*, Vol. 5; Gutierrez-Ramos et al., pp. 9171-9175, 1992, Vol. 89; Keller et al., pp. 473-486, *Mol. Cell. Biol.*, Vol 13; and 10 Breier et al., pp. 521-532, 1992, *Development*, Vol. 114. The use of ES cells to obtain endothelial populations of differentiated cells has been suggested by Wang et al., pp. 303-316, 1992, *Development*, Vol 114. Prior investigators, however, have failed to obtain populations of totipotent cells 15 (i.e., cells that can develop into any lineage, discussed in detail below) and pluripotent cells (i.e., cells, that while unable to develop into all lineages of cells, are at least able to develop into all hematopoietic lineages, also discussed in detail below). A reason for this failure is that 20 the ES cells were cultured under conditions in which the cells committed to a cellular lineage early in the tissue culture process. As a result, prior investigators failed to recognize a method for obtaining substantially homogeneous populations of totipotent or pluripotent embryonic cells that are useful 25 for therapeutic or experimental use. In addition, prior

investigators failed to recognize a method for inducing substantially homogeneous populations of totipotent or pluripotent embryonic cells to develop into preferred cell types.

5 Thus, there remains a need to develop a population of embryonic cells that are totipotent or pluripotent and, therefore, are capable of developing into a wide variety of cellular lineages.

SUMMARY OF THE INVENTION

10 The present invention relates to novel populations of totipotent embryonic cells that are capable of being developed into any cell type. The totipotent embryonic cells of the present invention can be used to derive additional populations of totipotent or pluripotent embryonic cells. The embryonic
15 cell populations of the present invention are particularly advantageous in that the populations provide useful reagents for therapeutic and experimental use.

One embodiment of the present invention is an embryoid body cell population that can be derived by culturing an
20 embryonic stem cell population in an embryoid body cell medium that includes platelet-poor fetal bovine serum, preferably from about 1 day to about 7 days. Such an embryoid body cell population includes cells of a variety of lineages, including cells of mesodermal cellular lineage, ectodermal cellular
25 lineage and endodermal cellular lineage. Also included in the

present invention is a method to obtain embryoid body cell populations from embryonic stem cells.

Another embodiment of the present invention is a method to produce mesodermal cells, ectodermal cells, or endodermal cells, as well as progenitors and progeny of such cells. The method includes the steps of (a) selecting a desired cell type; and (b) culturing an embryoid body cell population of the present invention under conditions suitable to obtain that cell type. Preferred cell types to obtain include those of hematopoietic, endothelial, muscle, epithelial and neural lineages.

Yet another embodiment of the present invention is an embryonic blast cell population that can be derived by culturing an embryoid body cell population under conditions effective to produce the embryonic blast cell population. Such conditions include an embryonic blast cell medium, which preferably includes at least one of the following growth factors: a hematopoietic cell growth factor and an endothelial cell growth factor. Embryonic blast cell populations are capable of developing into a variety of cell types, depending on the conditions under which the populations are cultured. Preferred cell types include primitive erythroid cells, definitive erythroid cells, macrophages, neutrophils, mast cells, T cells, endothelial cell, B cells, natural killer

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cells, megakaryocytes and eosinophils, as well as progenitors and progeny thereof.

The present invention also includes a method to obtain a population including embryonic blast cells. Such a method
5 includes the step of culturing a population of embryoid body cells under conditions effective to obtain embryonic blast cells including culturing in an embryonic blast cell medium. The step of culturing is preferably performed at a cell density of from about 1×10^5 cells to about 7.5×10^5 cells
10 per milliliter of culture medium, from about 2 days to about 15 days.

The present invention also includes a mixed population of endothelial cells and erythroid cells that can be derived by culturing an embryoid body cell population in an effective
15 medium that includes an endothelial cell growth factor and an erythroid cell growth factor. Also included in the present invention is a method to produce a mixed population of endothelial and erythroid cells that includes the step of culturing an embryoid body cell population in an effective
20 medium that includes an endothelial cell growth factor and an erythroid cell growth factor.

Another embodiment of the present invention is a BLAST-LYM cell population that can be derived by culturing an embryonic blast cell population in a BLAST-LYM cell medium
25 that includes one or more lymphoid cell growth factors. Also

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included in the present invention is a method to obtain a BLAST-LYM cell population that includes the step of culturing an embryonic blast cell population with a BLAST-LYM cell medium that includes one or more lymphoid growth factors.

5 In accordance with the present invention, a BLAST-LYM cell population can be further cultured in a fetal thymi culture to obtain a T cell population. Alternatively, a BLAST-LYM cell population of the present invention can be cultured with bone marrow stromal cells to produce lymphoid
10 cells having rearranged immunoglobulin genes. The present invention includes T cell populations and B cell populations obtained from BLAST-LYM cell populations, as well as methods to obtain such populations.

Yet another embodiment of the present invention is a
15 BLAST-NEM cell population that can be derived by culturing an embryonic blast cell population in an BLAST-NEM cell medium that includes a BLAST-NEM cell growth factor. A BLAST-NEM cell population is capable of developing into a variety of cell types such as, primitive erythroid cells, definitive
20 erythroid cells, macrophages, mast cells, neutrophils, eosinophils, and megakaryocytes, as well as progenitors and progeny thereof. Also included in the present invention is a method to obtain a BLAST-NEM cell population that includes the step of culturing an embryonic blast cell population in a
25 BLAST-NEM cell medium that includes a BLAST-NEM growth factor.



Another aspect of the present invention is a method to identify a compound expressed during the development of a population of embryonic blast cells. Such a method includes the step of characterizing the cellular composition of at least one cell contained in a population of cells selected from the group consisting of an embryonic stem cell population, an embryoid body cell population, an embryonic blast cell population, and intermediate cell populations thereof, to identify a compound expressed during the development of a population of embryonic blast cells. Such a compound is preferably capable of regulating at least one of the following cell functions: maintaining the survival of a cell, inducing the propagation of a cell, and stimulating the differentiation of a cell. Preferred compounds are nucleic acids, proteins, carbohydrates and lipids, with cell surface molecules, secreted molecules, cytoplasmic signal transduction molecules, and nucleic acid binding proteins being particularly preferred.

Another embodiment of the present invention is a formulation that includes culture medium isolated from one of the following: (a) a composition obtained by culturing a population of embryonic stem cells from about 1 day to about 7 days in an embryoid body cell medium to obtain an embryoid body cell population; and (b) a composition obtained by culturing a population of embryoid body cells for from about

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3 to about 6 days in an embryonic blast medium to obtain embryonic blast cells.

Ins' G1 *G1* BRIEF DESCRIPTION OF THE FIGURES

5 Fig. 1 is a schematic representation of the development of embryonic cell populations.

Fig. 2 shows a representative microscopic field of view of an embryoid body population and an embryonic blast cell population.

PA *A and b* Fig. 3_A illustrates cell surface marker staining of an
10 embryonic stem cell population, a Day 4 embryoid body population and a Day 6 embryonic blast cell population.

Fig. 4 shows a representative microscopic field of view of a mixed population of erythroid and endothelial cells.

15 Fig. 5 illustrates the kinetics of embryonic blast cell population development over time.

Fig. 6 is a schematic representation of a myeloid assay.

Fig. 7 illustrates the hematopoietic potential of embryonic blast cell populations.

20 Fig. 8 illustrates the effect of the age of embryoid body cell populations on multi-lineage embryonic blast cell population development.

Fig. 9 illustrates a model of hematopoiesis during ontogeny.

Fig. 10 illustrates the effect of specific growth factors on embryonic blast cell population development.

Fig. 11 illustrates T cell receptor expression by an embryonic stem cell-derived T cell population.

5 Fig. 12 illustrates the effect of specific growth factors on T cell receptor expression by an embryonic stem cell-derived T cell population.

Fig. 13 illustrates the effect of the age of an embryoid body cell population on the development of a mixed population
10 of erythroid and endothelial cells.

Fig. 14 illustrates the effect of specific growth factors on the development of a mixed population of erythroid and endothelial cells.

Fig. 15 illustrates the effect of the concentration of
15 specific growth factors on the development of a mixed population of erythroid and endothelial cells.

in Fig. 16^{A-C, A and B} shows representative microscopic fields of view of a mixed population of erythroid and endothelial cells stained with von Willebrand factor.

20 Fig. 17 illustrates the clonality of the development of a mixed population of erythroid and endothelial cells.

DETAILED DESCRIPTION OF THE INVENTION

One aspect of the present invention includes populations of embryonic cells that are capable of developing into

different cellular lineages when cultured under appropriate conditions. As used herein, the term "population" refers to one or more cells. The term "lineage" refers to all of the stages of the development of a cell type, from the earliest precursor cell to a completely mature cell (i.e., a specialized cell). A representation of the developmental pathways of populations of embryonic cells of the present invention is shown in Fig. 1.

Referring to Fig. 1 and in accordance with the present invention, a population of totipotent embryonic stem (ES) cells are allowed to differentiate and generate a population of pluripotent embryoid body (EB) cells in tissue culture. A population of pluripotent EB cells of the present invention can be dissociated and re-cultured to obtain two distinct populations of cells depending on the growth factors present in the culture medium. The first population includes pluripotent embryonic blast (BLAST) cells and the second population includes a mixed population of endothelial and erythroid cells. According to the present invention, the term "growth factor" is used in its broadest context and refers to all factors that are capable of stimulating the growth of a cell, maintaining the survival of a cell and/or stimulating the differentiation of a cell.

The population of BLAST cells can be further cultured in the presence of certain growth factors to obtain a population

of pre-lymphoid cells (i.e., cells capable of developing into lymphoid cells, such as T cells and B cells) referred to herein as BLAST-LYM cells. The population of blast cells can also be dissociated and re-cultured in the presence of certain growth factors to obtain a population of cells that includes erythrocytes and leukocytes other than lymphocytes. Cells in this population are referred to herein as BLAST-NEM cells. The developmental potential of the foregoing populations of cells indicate that the populations represent early stages of differentiation.

According to the present invention, the term "totipotent cell" refers to a cell capable of developing into all lineages of cells. Similarly, the term "totipotent population of cells" refers to a composition of cells capable of developing into all lineages of cells. Also according to the present invention, the term "pluripotent cell" refers to a cell capable of developing into a variety (albeit not all) lineages and are at least able to develop into all hematopoietic lineages (e.g., lymphoid, erythroid, and thrombocytic lineages). For example, a pluripotent cell can differ from a totipotent cell by having the ability to develop into all cell lineages except endothelial cells. A "pluripotent population of cells" refers to a composition of cells capable of developing into less than all lineages of cells but at least into all hematopoietic lineages. As such, a totipotent cell

or composition of cells is less developed than a pluripotent cell or compositions of cells. As used herein, the terms "develop", "differentiate" and "mature" all refer to the progression of a cell from the stage of having the potential to differentiate into at least two different cellular lineages to becoming a specialized cell. Such terms can be used interchangeably for the purposes of the present application.

One aspect of the present invention is a method to produce a pluripotent population of EB cells that, when cultured under appropriate conditions, are capable of developing into a variety of cell lineages, including endothelial cell or hematopoietic lineage. A pluripotent EB cell population of the present invention can be derived by culturing a population of totipotent ES cells in an embryoid body medium including platelet-poor fetal bovine serum (PP-FBS). According to the present invention, PP-FBS refers to fetal bovine serum not having inhibitors of ES cell differentiation (e.g., TGF- β). A preferred PP-FBS of the present invention comprises fetal bovine blood from which platelets have been removed and the resulting plasma has been clotted, thereby producing platelet-poor serum. Suitable ES cells for use with the present invention include inner mass cells derived from an about 3.0 day old to about 4.0 day old blastocyst, with a blastocyst about 3.5 days old being more preferred. ES cells of the present invention are derived from

an animal, preferably from a mammal, and more preferably from a human, mouse, primate, pig, cow, sheep, rabbit, rat, guinea pig or hamster.

In one embodiment, an EB cell population of the present invention is derived by culturing a population of ES cells in an embryoid body medium, which is medium that stimulates the differentiation of an ES cell population to an EB cell population. Typically, an ES cell population is maintained in an undifferentiated state by culturing the cells in an embryonic stem cell medium including leukemia inhibitory factor (LIF) and fetal calf serum (FCS). To produce an EB cell population in accordance with the present invention, an ES cell population is removed from the embryonic stem cell medium and re-cultured in embryoid body medium in which the LIF and the FCS have been replaced by PP-FBS. Both the absence of LIF and the presence of PP-FBS in the culture medium stimulates the ES cell population to differentiate into an EB cell population of the present invention. An embryoid body medium of the present invention includes a suitable amount of PP-FBS that is capable of stimulating the differentiation of an ES cell population to an EB cell population. A preferred embryoid body cell medium of the present invention includes from about 5% to about 30%, more preferably from about 10% to about 20%, and even more preferably about 15% PP-FBS.

Applicants have discovered that culturing of an ES cell population for a certain period of time results in the differentiation of the ES cell population to an EB cell population in which the EB cells are pluripotent. If cultured
5 for too long, as has been done by prior investigators, the EB cell population loses pluripotency. As such, in accordance with the present invention, an EB cell population of the present invention is derived by culturing a population of ES cells for a suitable amount of time to produce a pluripotent
10 population of EB cells. In other words, an EB cell population of the present invention is derived by culturing a population of ES cells for an amount of time that maintains an EB cell population at a stage of pluripotency. In particular, the present invention includes a population of EB cells that are
15 derived by culturing a population of ES cells for a suitable amount of time to produce a population of EB cells that is capable of developing into an endothelial cell lineage and/or a hematopoietic cell lineage. In accordance with the present invention, an EB cell population is derived by culturing a
20 population of ES cells from about 1 day to about 7 days. A preferred EB cell population of the present invention is derived by culturing a population of ES cells from about 3 days to about 4 days, with from about 72 hours to 96 hours being more preferred.

In accordance with the present invention, the culture conditions are also important in obtaining a pluripotent EB cell population of the present invention from a totipotent population of ES cells. For example, an ES cell population is
5 cultured in suspension to derive an EB cell population. During culturing, variables such as cell density, temperature and CO₂ levels can be controlled to maximize the development of populations of EB cells. For example, it appears that the density of cells in an ES cell culture can affect the
10 development of an EB cell population. While not being bound by theory, it is believed that ES cell populations produce one or more growth factors that are capable of stimulating the differentiation of the ES cell population into an EB cell population. As such, the optimum cell density for the growth
15 of an EB cell population is from about 1×10^3 ES cells per ml to about 100×10^3 ES cells per ml, more preferably from about 2×10^3 ES cells per ml to about 10×10^3 ES cells per ml, and even more preferably from about 3×10^3 ES cells per ml to about 4.5×10^3 ES cells per ml. The optimum temperature for
20 the development of an EB cell population is from about 35°C and about 39°C, preferably from about 36°C and 38°C, with a temperature of 37°C being even more preferred. The optimum CO₂ levels in the culturing environment for the development of EB cell populations is from about 3% CO₂ to about 10% CO₂,

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more preferably from about 4% CO₂ to about 6% CO₂, and even more preferably about 5% CO₂.

In a preferred embodiment, an EB cell population of the present invention is derived by culturing a population of ES
5 cells in an embryoid body medium including Iscove's Modified Dulbecco's Medium (IMDM), with about 15% PP-FBS (obtained from Antech, TEXAS), monothiolglycerol (MTG), transferrin, glutamine, at a cell density of about 4.5×10^3 cells per ml of medium. The ES cell population is then cultured for about
10 96 hours, at about 37°C, in an about 5% CO₂-containing environment to obtain a population of pluripotent EB cells.

An EB cell population of the present invention is capable of developing into cells of mesodermal cell lineage, of ectodermal cell lineage or of endodermal cell lineage. As
15 used herein, mesodermal cells include cells of connective tissue, bone, cartilage, muscle, blood and blood vessel, lymphatic and lymphoid organ, notochord, pleura, pericardium, peritoneum, kidney and gonad. Ectodermal cells include epidermal tissue cells, such as those of nail, hair, glands of
20 the skin, the nervous system, the external sense organs (e.g., eyes and ears) and mucous membranes (such as those of the mouth and anus). Endodermal cells include cells of the epithelium such as those of the pharynx, respiratory tract (except the nose), digestive tract, bladder and urethra cells.
25 Preferred cells within an EB cell population of the present

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invention include cells of at least one of the following cellular lineages: hematopoietic cell lineage, endothelial cell lineage, epithelial cell lineage, muscle cell lineage and neural cell lineage. More preferred cells within an EB cell population of the present invention include cells of erythroid lineage, endothelial lineage, leukocyte lineage and thrombocyte lineage. Even more preferred cells within an EB cell population of the present invention include cells of erythroid lineage (including primitive and definitive erythroid lineages), macrophage lineage, neutrophil lineage, mast cell lineage, megakaryocyte lineage, natural killer cell lineage, eosinophil lineage, T cell lineage, endothelial cell lineage and B cell lineage.

An EB cell population of the present invention includes a colony of cells having substantially the same morphology as the colony of cells shown in Fig. 2, cell colony A. The EB cell population shown in Fig. 2, cell colony A, was obtained when ES cells were grown as described in Example 1. Referring to Fig. 2, the EB cell population shown as cell colony A has a general morphology of tightly packed cells, in which individual cells are not easily detectable.

An EB cell population of the present invention that has been derived by culturing a population of ES cells for 4 days (i.e., Day 4 EB) can include cells that have substantially the same antibody staining pattern as shown in Fig. 3A and 3B,

when such EB cells are stained according to the method described in Example 2. Referring to Fig. 3A and 3B, a Day 4 EB cell population expresses substantially low amounts of Sca-1, C-kit receptor and Class I H-2b, and essentially no Thy 1, VLA-4, CD44 and CD45.

Another aspect of the present invention is a method to produce a cell type, such as a mesodermal cell, an ectodermal cell and/or an endodermal cell that includes the steps of: (a) selecting a desired cell type to produce; and (b) culturing an embryoid body cell population of the present invention under conditions suitable to obtain the desired cell type. Suitable culture conditions for obtaining a desired cell type include culturing the EB cell population in a medium including a growth factor that is able to stimulate the EB cell population to differentiate to the desired cell type(s). As used herein, the term "a" refers to "at least one", or "one or more." For example, an EB cell population can be cultured in a medium including a growth factor capable of promoting the differentiation of the cell population into a hematopoietic cell type. A preferred culture condition for obtaining a desired cell type that includes erythroid and endothelial cells includes culturing an EB cell population of the present invention in the presence of erythropoietin (EPO) and vascular endothelial growth factor (VEGF; described in detail below). Another preferred culture condition for obtaining a desired

cell type of embryonic blast cells includes culturing an EB cell population of the present invention in the presence of C-kit ligand, interleukin 1 (IL-1), interleukin 3 (IL-3), interleukin 6 (IL-6), interleukin 11 (IL-11), EPO, VEGF, and mixtures thereof, to obtain a cell of a particular cell type (described in detail below).

In the mouse, the first visible signs of blood cell development are the appearance of foci or red blood cells in a tissue called the yolk sac at 7.5 days of gestation. These early appearing red blood cells are primitive erythroid cells. Cells which are destined to make blood vessels, the endothelial cells, appear at almost the same time and in the same location as such embryonic red blood cells. Without being bound by theory, it is believed that the parallel appearance of the two types of cells in close proximity indicates that the two cell types develop from a common precursor. Prior investigators, however, have merely theorized about the existence of such a precursor cell population and have not taught or enabled the isolation of the population. Applicants have identified for the first time a population of precursor cells that are able to develop into endothelial and erythroid lineages.

One aspect of the present invention is a method to produce a mixed population of erythroid and endothelial cells by culturing a population of pluripotent EB cells of the

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present invention in an endothelial/erythroid cell medium, which is medium that stimulates the differentiation of an EB cell population of the present invention to a population of erythroid and endothelial cells. An endothelial/erythroid cell medium of the present invention includes a suitable amount of a growth factor capable of stimulating the development of an EB cell population into a mixed population of endothelial cells and erythroid cells. A preferred endothelial/erythroid cell medium of the present invention includes a hematopoietic cell growth factor, an endothelial cell growth factor, homologues of such growth factors, or mixtures of such growth factors and/or homologues. A more preferred endothelial/erythroid cell medium of the present invention includes C-kit ligand, EPO and VEGF, homologues of such growth factors, or mixtures of such growth factors and/or homologues. An even more preferred cell medium of this embodiment of the present invention includes EPO and VEGF.

According to the present invention, an endothelial/erythroid cell medium of the present invention includes a suitable growth factor and PP-FBS. A preferred endothelial/erythroid cell medium of the present invention includes from about 5% to about 30%, more preferably from about 7% to about 20%, and even more preferably about 10% PP-FBS.

Also according to the present invention, an EB cell population of the present invention is preferably cultured in methyl cellulose to obtain a mixed population of endothelial and erythroid cells. A suitable amount of methyl cellulose for culturing EB cell populations is an amount that enables the EB cells to associate as groups (i.e., clumps or clusters) of cells, thereby stimulating growth and/or differentiation of the EB cells into cells. A preferred amount of methyl cellulose in which to culture an EB cell population of the present invention to obtain a mixed population of endothelial and erythroid cells is from about 0.25% to about 2%, more preferably from about 0.5% to about 1.5%, and even more preferably at about 1%.

Also according to the present invention, an EB cell population of the present invention is cultured in an endothelial/erythroid cell medium for a sufficient amount of time to allow the EB cell population to differentiate to a mixed population of endothelial and erythroid cells. A preferred amount of time to culture an EB cell population is from about 5 days to about 12 days. A more preferred amount of time to culture an EB cell population is from about 6 days to about 11 days. An even more preferred amount of time to culture an EB cell population is from about 7 days to about 10 days.

Other culture conditions (i.e., in addition to time and medium) which can effect the development of a mixed population of endothelial and erythroid cells of the present invention from an EB cell population of the present invention includes
5 the temperature and CO₂ content of the culture environment. The optimum temperature for the development of a mixed population of endothelial and erythroid cells of the present invention is from about 35°C to about 39°C, preferably from about 36°C to 38°C, with a temperature of 37°C being even more
10 preferred. The optimum CO₂ levels in the culturing environment for the development of a mixed population of endothelial and erythroid cells is from about 3% CO₂ to about 10% CO₂, more preferably from about 4% CO₂ to about 6% CO₂, and even more preferably about 5% CO₂.

15 A mixed population of endothelial and erythroid cells of the present invention is derived by culturing a population of EB cells at a suitable cell density to produce a mixed population of endothelial and erythroid cells. The optimum cell density for the growth of the population is preferably
20 from about 5×10^4 cells to about 7.5×10^5 EB cells, more preferably from about 1.5×10^5 cells to about 6×10^5 EB cells, and even more preferably from about 2×10^5 cells to about 5×10^5 EB cells per ml of culture medium.

In a preferred embodiment, a mixed population of
25 endothelial and erythroid cells of the present invention is

derived by culturing a population of EB cells of the present invention in an endothelial/erythroid cell medium including IMDM, with about 10% PP-FBS, 1% methyl cellulose, and a mixture of growth factors including VEGF and EPO for about 7
5 days, at about 37°C, in an about 5% CO₂-containing environment to obtain a mixed population of endothelial and erythroid cells.

In one embodiment, a mixed population of endothelial and erythroid cells includes one or more cells of endothelial
10 lineage or erythroid lineage. A preferred mixed population of endothelial and erythroid cells includes one or more cells that can be stained with von Willebrand factor according to the method described in Example 10, and/or one or more cells that can absorb diI-acetylated-low density lipoproteins when
15 labelled according to the method described in Example 10.

A mixed population of endothelial and erythroid cells of the present invention includes one or more cells having a substantially similar morphology as the cells shown in Fig. 4. Referring to Fig. 4, generally 3 types of cells having
20 different morphologies can be found in a mixed population of endothelial and erythroid cells of the present invention. A first cell type, indicated as cell A in Fig. 4, comprises an erythroid cell having the typical characteristics of a distinct compact cluster of small cells having red color. A
25 second cell type, indicated as cell B in Fig. 4, comprises a

spherical cell having a larger size than an erythroid cell, such as cell A. A third cell type, indicated as cell C in Fig. 4, comprises a spherical cell having a similar size as an erythroid cell but having a single long process extending from the cell. According to the present invention, both the second cell type (i.e., cell B in Fig. 4) and the third cell type (i.e., cell C in Fig. 4) can be stained with von Willebrand factor and can absorb diI-acetylated-low density lipoproteins, thereby indicating that such cell types are representative of endothelial cells.

Another aspect of the present invention is a method to produce a pluripotent population of BLAST cells that, while unable to develop into all lineages of cells, are at least able to develop into all hematopoietic lineages, when cultured under appropriate conditions. In one embodiment, a pluripotent BLAST cell population of the present invention can be derived by culturing a population of pluripotent EB cells of the present invention in an embryonic blast cell medium, which is medium that stimulates the differentiation of an EB cell population of the present invention to a BLAST cell population of the present invention. An embryonic blast cell medium of the present invention includes a suitable amount of a growth factor capable of stimulating the development of an EB cell population into a pluripotent BLAST cell population. A preferred embryonic blast cell medium of the present

invention includes a hematopoietic cell growth factor, an endothelial cell growth factor or a mixture thereof. As used herein, a homologue of a specific growth factor refers to a compound that is capable of having substantially similar activity as that growth factor; i.e., a homologue of a growth factor is substantially similar to that growth factor. For example, a homologue of a specific growth factor can bind to the cell surface receptor of that growth factor in such a manner that the surface receptor is stimulated to induce an appropriate cellular function similar to that effected by the specific growth factor. A more preferred embryonic blast cell medium of the present invention includes C-kit ligand, IL-1, IL-3, IL-6, IL-11, EPO, VEGF, homologues of such growth factors, or mixtures of such growth factors and/or homologues. An even more preferred embryonic blast cell medium of the present invention includes a mixture of C-kit ligand, IL-1, IL-6 and IL-11; a mixture of C-kit ligand, EPO and VEGF; or C-kit ligand alone.

In accordance with the present invention, an embryonic blast cell medium includes PP-FBS in addition to one or more growth factors described above. A preferred embryonic blast cell medium of the present invention includes from about 5% and about 20%, more preferably from about 7% to about 15%, and even more preferably about 10% PP-FBS.

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Also according to the present invention, an EB cell population of the present invention is cultured in methyl cellulose to obtain a population of BLAST cells of the present invention. A suitable amount of methyl cellulose for culturing EB cell populations is an amount that enables the EB cells to associate as groups (i.e., clumps or clusters) of cells, thereby stimulating growth and/or differentiation of the EB cells into BLAST cells. A preferred amount of methyl cellulose in which to culture an EB cell population of the present invention is from about 0.25 % to about 2.0%, more preferably from about 0.5% to about 1.5%, and even more preferably at about 1%.

A BLAST cell population of the present invention is derived by culturing a population of EB cells at a suitable cell density to produce a pluripotent population of BLAST cells. The optimum cell density for the growth of a BLAST cell population is preferably from about 1×10^5 cells to about 7.5×10^5 EB cells, more preferably from about 1.5×10^5 cells to about 6×10^5 EB cells, and even more preferably from about 2×10^5 cells to about 5×10^5 EB cells per ml of culture medium.

Applicants have discovered that culturing of an EB cell population for a certain period of time in accordance with the present invention results in the differentiation of an EB cell population to a BLAST cell population in which the BLAST cells

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are pluripotent. If cultured for too long, as has been done by prior investigators, the BLAST cell population loses pluripotency. As such, a BLAST cell population of the present invention is derived by culturing a population of EB cells for a suitable amount of time to produce a pluripotent population of BLAST cells. In particular, a population of EB cells are cultured for a suitable amount of time to produce a population of BLAST cells that is capable of developing into any hematopoietic cell lineage. In other words, a BLAST cell population of the present invention is derived by culturing a population of EB cells for an amount of time that maintains a BLAST cell population at a stage of pluripotency. A preferred BLAST cell population is derived by culturing a population of EB cells from at least about 2 days to about 15 days. A more preferred BLAST cell population of the present invention is derived by culturing a population of EB cells from about 3 days to about 10 days. An even more preferred BLAST cell population of the present invention is derived by culturing a population of EB cells from about 3 days to about 6 days.

Other culture conditions (i.e., in addition to time and medium) which can effect the development of a BLAST cell population of the present invention from an EB cell population of the present invention include the temperature and CO₂ content of the culture environment. The optimum temperature for the development of a BLAST cell population of the present

invention is from about 35°C to about 39°C, preferably from about 36°C to about 38°C, with a temperature of about 37°C being even more preferred. The optimum CO₂ levels in the culturing environment for the development of BLAST cell populations is from about 3% CO₂ to about 10% CO₂, more preferably from about 4% CO₂ to about 6% CO₂, and even more preferably about 5% CO₂.

In a preferred embodiment, a BLAST cell population of the present invention is derived by culturing a population of EB cells of the present invention in an embryonic blast cell medium including IMDM, with about 10% PP-FBS, 1% methyl cellulose, and either a mixture of growth factors including IL-1, IL-6, IL-11, C-kit ligand, EPO and VEGF, or C-kit ligand alone. The EB cell population is grown a cell density of from about 2 x 10⁵ cells per ml of medium to about 5 x 10⁵ cells per ml of medium. After reaching that density, the EB cell population is then cultured for about 6 days, at about 37°C, in an about 5% CO₂-containing environment to obtain a population of pluripotent BLAST cells.

Being pluripotent, a BLAST cell population of the present invention includes cells of hematopoietic and other cell lineages. In particular, a BLAST cell population of the present invention includes cells of erythroid lineage, endothelial lineage, leukocyte lineage and thrombocyte lineage. A preferred BLAST cell population of the present

invention includes cells capable of developing into primitive erythroid cells, definitive erythroid cells, macrophages, neutrophils, mast cells, T cells, endothelial cell, B cells, natural killer cells, megakaryocytes, eosinophils, and progenitors and progeny thereof. As used herein, a primitive erythroid cell is characterized by the cell's nucleated morphology and expression of embryonic globin. A definitive erythroid cell (also referred to as an adult erythroid cell) is characterized by the cell's expression of adult globin and eventual enucleation. As used herein, a "progenitor" cell refers to an ancestor of a cell (i.e., a cell from which a subject cell is derived). As used herein, a "progeny" cell refers to a cell derived from a subject cell.

A BLAST cell population of the present invention includes a colony of cells having substantially the same morphology as the colony of cells shown in Fig. 2, cell colony B. The BLAST cell population shown in Fig. 2, cell colony B was obtained when EB cells were grown as described in Example 3. Referring to Fig. 2, the BLAST cell population shown as cell colony B has a general morphology of clumped, but not tightly packed cells, in which individual cells can be discerned when the colony is viewed under the microscope.

A BLAST cell population of the present invention that has been derived by culturing a population of EB cells for 6 days (i.e., Day 6 BLASTS) can include cells that have substantially

the same antibody staining pattern as shown in Fig. 3A and 3B, when such BLAST cells are stained according to the method described in Example 4. Referring to Fig. 3A and 3B, a Day 6 BLAST cell population expresses substantial amounts of CD44, C-kit receptor, Sca-1 and VLA-4, and essentially no Class I H-2b, Thy 1 and CD45.

Another aspect of the present invention is a method to produce a population of BLAST-LYM cells that are able to develop into a cell of lymphoid lineage, when cultured under appropriate conditions. In one embodiment, a BLAST-LYM cell population of the present invention can be derived by culturing a population of pluripotent BLAST cells of the present invention in a BLAST-LYM cell medium, which is medium that stimulates the differentiation of a BLAST cell population of the present invention to a BLAST-LYM cell population of the present invention. A BLAST-LYM cell medium of the present invention includes a suitable amount of one or more lymphoid cell growth factors that are capable of stimulating the differentiation of a BLAST cell population to an BLAST-LYM cell population. A preferred BLAST-LYM cell medium for the production of BLAST-LYM cells of the present invention includes one or more of the lymphoid growth factors IL-7, C-kit ligand, insulin-like growth factor 1 (IGF-1), VEGF, EPO, a growth factor produced by an embryoid body cell, homologues of such growth factors, or mixtures of such growth factors

and/or homologues. A more preferred BLAST-LYM cell medium for the production of BLAST-LYM cells of the present invention includes one or more of the lymphoid growth factors IL-7, C-kit ligand, insulin-like growth factor 1 (IGF-1), homologues
5 of such growth factors, or mixtures of such growth factors and/or homologues.

According to the present invention, a BLAST-LYM cell medium of the present invention includes PP-FBS as well as one or more suitable growth factor as described above. A
10 preferred BLAST-LYM cell medium of the present invention includes from about 5% to about 30%, more preferably from about 7% to about 20%, and even more preferably about 10% PP-FBS.

Also according to the present invention, a BLAST cell
15 population of the present invention is preferably cultured in methyl cellulose to obtain a population of BLAST-LYM cells. A suitable amount of methyl cellulose for culturing BLAST cell populations is an amount that enables the BLAST-LYM cells to associate as groups (i.e., clumps or clusters) of cells,
20 thereby stimulating growth and/or differentiation of the BLAST cells into BLAST-LYM cells. A preferred amount of methyl cellulose in which to culture a BLAST cell population of the present invention to obtain a BLAST-LYM is from about 0.25% to about 2.0%, more preferably from about 0.5% to about 1.5%, and
25 even more preferably at about 1%.

A BLAST-LYM cell population of the present invention is derived by culturing a population of BLAST cells for a suitable amount of time to produce a BLAST-LYM cell population able to develop into a lymphoid lineage. In particular, the present invention includes a population of BLAST-LYM cells that are derived by culturing a population of BLAST cells for a suitable amount of time to produce a population of BLAST-LYM cells that are capable of developing into a T cell or a B cell when cultured under appropriate conditions. According to the present invention, a T cell includes a cell that represents a given stage of T cell maturation, and as such, can rearrange from immature T cells having rearranged T cell receptor germline genes to mature T cells expressing $\alpha\beta$ T cell receptor proteins. Also according to the present invention, a B cell can include a cell that represents a given stage of B cell maturation, and as such, can range from an early B cell having rearranged diversity (i.e., D) region and joining (i.e., J) region immunoglobulin germline genes, more preferably a cell having rearranged diversity D, J and variable (i.e., V) region immunoglobulin germline genes, to a plasma cell that is able to secrete immunoglobulin proteins. A BLAST-LYM cell population is derived by culturing a population of BLAST cells from about 3 day to about 10 days, preferably for about 6 days.

In accordance with the present invention, other culture conditions (i.e., in addition to time and medium) are also important in obtaining a BLAST-LYM cell population of the present invention from a population of BLAST cells. During
5 culturing, variables such as cell density, temperature and CO₂ levels can be controlled to maximize the development of populations of BLAST-LYM cells. For example, it appears that the density of cells in a BLAST cell culture can affect the development of a BLAST-LYM cell population. The optimum cell
10 density for the growth of an BLAST-LYM cell population is from about 5×10^4 BLAST cells per ml to about 7.5×10^5 BLAST cells per ml, more preferably from about 1×10^5 BLAST cells per ml to about 6×10^5 BLAST cells per ml, and even more preferably from about 2.5×10^5 BLAST cells per ml to about 5
15 $\times 10^5$ BLAST cells per ml. The optimum temperature for the development of an BLAST-LYM cell population is from about 35°C to about 39°C, preferably from about 36°C to 38°C, with a temperature of 37°C being even more preferred. The optimum CO₂ levels in the culturing environment for the development of
20 BLAST-LYM cell populations is from about 3% CO₂ to about 10% CO₂, more preferably from about 4% CO₂ to about 6% CO₂, and even more preferably about 5% CO₂.

In a preferred embodiment, a BLAST-LYM cell population of the present invention is derived by culturing an individual
25 BLAST cell colony in a medium including IMDM, with about 10%

PP-FBS, 1% methyl cellulose, and a mixture of growth factors including IL-7, IGF-1 and C-kit ligand for about 6 days at about 37°C, in an about 5% CO₂-containing environment to obtain a population of BLAST-LYM cells.

5 In accordance with the present invention, a BLAST-LYM cell population of the present invention includes cells of a lymphoid lineage. Preferred cells within a BLAST-LYM cell population of the present invention include cells of a T cell lineage, a B cell lineage, and/or a natural killer cell
10 lineage. More preferred cells within a BLAST-LYM cell population of the present invention can develop into T cells having rearranged T cell receptor germline genes and B cells having rearranged D and J region immunoglobulin germline genes. Particularly preferred cells within a BLAST-LYM cell
15 population of the present invention can develop into a T cell expressing T cell receptor proteins, such as $\alpha\beta$ and/or $\gamma\delta$ T cell receptors, or a B cell having rearranged V, D and J region immunoglobulin germline genes.

One aspect of the present invention is a method to
20 produce a lymphoid cell population that includes the steps of:
(a) culturing a BLAST cell population in an BLAST-LYM cell medium including one or more lymphoid cell growth factors to produce a BLAST-LYM cell population; and (b) culturing the BLAST-LYM cell population with cells selected from the group

consisting of fetal thymi culture cells and bone marrow stromal cells to obtain a lymphoid cell population.

In one embodiment, a BLAST-LYM cell population of the present invention is cultured in a fetal thymi culture to
5 obtain a population of T cells. Preferably, a BLAST cell population cultured in the presence of C-kit ligand, IGF-1 and IL-7 is used to produce a BLAST-LYM cell which is cultured in a fetal thymi culture to produce a T cell population. Techniques to perform fetal thymi cultures are well known to
10 those of skill in the art. Preferably, a BLAST-LYM population is cultured in a fetal thymi culture for from about 1 week to about 6 weeks, more preferably for from about 1.5 weeks to about 4 weeks, and even more preferably for from about 2 weeks to about 3 weeks. A preferred T cell population of the
15 present invention is a population of cells comprising from about 1% to about 75% T cells, more preferably from about 3% to about 65% T cells, and even more preferably from about 5% to about 50% T cells. It is within the scope of the invention, however, that a T cell population can also include
20 other lymphocyte subpopulations that are typically found in a thymic population. For example, a T cell population of the present invention can include macrophages, dendritic cells, natural killer cells and epithelial cells. The T cells included in a T cell population of the present invention
25 preferably include T cells having rearranged T cell receptor

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germline genes and more preferably include T cells expressing T cell receptor proteins, including $\alpha\beta$ and/or $\gamma\delta$ T cell receptors.

In another embodiment, a BLAST-LYM cell population of the present invention is cultured in the presence of bone marrow stromal cells to obtain a population of B cells. Preferably, a BLAST cell population cultured in the presence of C-kit ligand alone is used to produce a BLAST-LYM cell which is cultured with bone marrow stromal cells to produce a B cell population. Techniques to perform bone marrow stromal cell cultures are well known to those of skill in the art. Preferably, a BLAST-LYM population is cultured in the presence of bone marrow stromal cells for from about 3 days to about 75 days, more preferably for from about 7 days to about 45 days, and even more preferably for from about 14 days to about 21 days. A preferred B cell population of the present invention is a population of cells comprising from about 0.5% to about 20% B cells, more preferably from about 0.75% to about 17% B cells, and even more preferably from about 1% to about 15% B cells. A preferred B cell population includes B cells having rearranged D and J region immunoglobulin germline genes, and more preferably B cells having rearranged V, D and J region germline genes.

Another aspect of the present invention is a method to produce a population of BLAST-NEM cells that are able to

develop into a cell of certain hematopoietic lineages when cultured under appropriate conditions. According to the present invention, a cell of hematopoietic lineage is able to develop into erythrocyte cells (i.e., a red blood cell),
5 certain leukocyte cells (i.e., a white blood cell other than lymphocytes), or thrombocyte cells (i.e., platelet cell). Leukocyte cells include granular leukocytes, including eosinophils, basophils, neutrophils, and mast cells; as well as non-granular leukocytes, including megakaryocytes,
10 polymorphonuclear cells, lymphocytes and monocytes (i.e., macrophages). A BLAST-NEM cell population of the present invention comprises cells that are able to develop into any hematopoietic cell type other than lymphocytes. A preferred BLAST-NEM cell population of the present invention includes
15 cells that are able to develop into erythrocytes, leukocytes other than lymphocytes, or thrombocytes. A more preferred BLAST-NEM cell population of the present invention includes cells that are able to develop into primitive erythroid cells, definitive erythroid cells, macrophages, mast cells,
20 neutrophils, eosinophils, megakaryocytes, undifferentiated hematopoietic cell colonies, or progenitors or progeny thereof. An even more preferred BLAST-NEM cell population of the present invention includes cells that are able to develop into primitive erythroid cells, definitive erythroid cells,

macrophages, mast cells, neutrophils, or progenitors or progeny thereof.

In one embodiment, a BLAST-NEM cell population of the present invention can be derived by culturing a population of pluripotent BLAST cells of the present invention in a BLAST-NEM cell medium, which is a medium that stimulates the differentiation of a BLAST cell population of the present invention to a BLAST-NEM cell population of the present invention. An BLAST-NEM cell medium of the present invention includes a suitable amount of one or more BLAST-NEM cell growth factors that are capable of stimulating the differentiation of a BLAST cell population to an BLAST-NEM cell population. For purposes of this application, BLAST-NEM cell growth factors differ from hematopoietic growth factors in that hematopoietic growth factors of the present invention include lymphoid factors and the BLAST-NEM cell growth factors of the present invention do not. A preferred BLAST-NEM cell medium includes one or more of the BLAST-NEM cell growth factors C-kit ligand, IL-1, IL-3, IL-6, IL-11, VEGF, EPO, homologues of such growth factors, or mixtures of such growth factors and/or homologues. A more preferred BLAST-NEM cell medium includes C-kit ligand, IL-1, IL-3, IL-6, IL-11, VEGF and EPO.

According to the present invention, a BLAST-NEM cell medium of the present invention includes PP-FBS as well as one

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or more suitable growth factor as described above. A preferred BLAST-NEM cell medium of the present invention includes from about 5% to about 30%, more preferably from about 7% to about 20%, and even more preferably about 10% PP-
5 FBS.

Also according to the present invention, a BLAST cell population of the present invention is preferably cultured in methyl cellulose to obtain a population of BLAST-NEM cells. A suitable amount of methyl cellulose for culturing BLAST cell
10 populations is an amount that enables the BLAST-NEM cells to associate as groups (i.e., clumps or clusters) of cells, thereby stimulating growth and/or differentiation of the BLAST cells into BLAST-NEM cells. A preferred amount of methyl cellulose in which to culture a BLAST cell population of the
15 present invention to obtain a BLAST-NEM is from about 0.25% to about 2.0%, more preferably from about 0.5% to about 1.5%, and even more preferably at about 1%.

A BLAST-NEM cell population of the present invention is derived by culturing a population of BLAST cells for a
20 suitable amount of time to produce a BLAST-NEM cell population able to develop into a hematopoietic lineage. In particular, the present invention includes a population of BLAST-NEM cells that are derived by culturing a population of BLAST cells for a suitable amount of time to produce a population of BLAST-NEM
25 cells that are capable of developing into erythrocyte or

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leukocyte cells. A BLAST-NEM cell population is derived by culturing a population of BLAST cells from about 2 days to about 12 days. A preferred BLAST-NEM cell population is derived by culturing a population of BLAST cells from about 4
5 days to about 8 days, with culturing for about 6 days being more preferred.

In accordance with the present invention, other culture conditions (i.e., in addition to time and medium) are also important in obtaining a BLAST-NEM cell population of the
10 present invention from a population of BLAST cells. During culturing, variables such as cell density, temperature and CO₂ levels can be controlled to maximize the development of populations of BLAST-NEM cells. For example, it appears that the density of cells in a BLAST cell culture can affect the
15 development of a BLAST-NEM cell population. The optimum cell density for the growth of an BLAST-NEM cell population is from about 5×10^4 BLAST cells per ml to about 7.5×10^5 BLAST cells per ml, more preferably from about 1×10^5 BLAST cells per ml to about 6×10^5 BLAST cells per ml, and even more
20 preferably from about 2×10^5 BLAST cells per ml to about 5×10^5 BLAST cells per ml. The optimum temperature for the development of an BLAST-NEM cell population is from about 35°C to about 39°C, preferably from about 36°C to 38°C, with a temperature of 37°C being even more preferred. The optimum
25 CO₂ levels in the culturing environment for the development of

BLAST-NEM cell populations is from about 3% CO₂ to about 10% CO₂, more preferably from about 4% CO₂ to about 6% CO₂, and even more preferably about 5% CO₂.

In a preferred embodiment, a BLAST-NEM cell population of
5 the present invention is derived by culturing a population of
BLAST cells in an embryoid body medium including IMDM, with
about 10% PP-FBS, 1% methyl cellulose, and a mixture of growth
factors including C-kit ligand, IL-1, IL-3, IL-6, IL-11, VEGF
and EPO for about 6 days at about 37°C, in an about 5% CO₂-
10 containing environment to obtain a population of BLAST-NEM
cells.

Another aspect of the present invention is a method to
produce a population of leukocytes and erythrocytes that
includes the steps of: (a) culturing a BLAST cell population
15 in an BLAST-NEM cell medium including one or more BLAST-NEM
cell growth factors to produce a BLAST-NEM cell population;
and (b) culturing the BLAST-NEM cell population with one or
more leukocyte and/or erythrocyte growth factors to obtain a
mixed population of leukocyte and/or erythrocyte cells.
20 Preferred leukocyte and/or erythrocyte growth factors useful
for the production of a population of leukocytes and
erythrocytes include one or more of the leukocyte and/or
erythrocyte growth factors C-kit ligand, IL-1, IL-3, IL-6, IL-
11, EPO, GM-CSF, G-CSF, M-CSF, homologues of such growth
25 factors, or mixtures of such growth factors and/or homologues.

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More preferred leukocyte and/or erythrocyte growth factors include C-kit ligand, IL-1, IL-3, IL-6, IL-11, EPO, GM-CSF, G-CSF and/or M-CSF.

A mixed population of leukocyte and/or erythrocyte cells is derived by culturing a population of BLAST-NEM cells from about 2 days to about 14 days. A preferred mixed population of leukocyte and/or erythrocyte cells is derived by culturing a population of BLAST-NEM cells from about 4 days to about 12 days, with culturing for about 8 days being more preferred.

According to the present invention, a BLAST-NEM cell medium of the present invention includes PP-FBS as well as one or more suitable growth factor as described above. A preferred BLAST-NEM cell medium of the present invention includes from about 5% to about 30%, more preferably from about 7% to about 20%, and even more preferably about 10% PP-FBS.

Also according to the present invention, a BLAST cell population of the present invention is cultured in methyl cellulose to obtain a population of BLAST-NEM cells. A suitable amount of methyl cellulose for culturing BLAST cell populations is an amount that enables the BLAST-NEM cells to associate as groups (i.e., clumps or clusters) of cells, thereby stimulating growth and/or differentiation of the BLAST cells into BLAST-NEM cells. A preferred amount of methyl cellulose in which to culture a BLAST cell population of the

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present invention to obtain a BLAST-NEM is from about 0.25% to about 2.0%, more preferably from about 0.5% to about 1.5%, and even more preferably at about 1%.

A BLAST-NEM cell population of the present invention is derived by culturing a population of BLAST cells for a suitable amount of time to produce a BLAST-NEM cell population able to develop into a hematopoietic lineage. In particular, the present invention includes a population of BLAST-NEM cells that are derived by culturing a population of BLAST cells for a suitable amount of time to produce a population of BLAST-NEM cells that are capable of developing into a primitive erythroid cell, a definitive erythroid cell, a macrophage, a neutrophil or a mast cell, when cultured under appropriate conditions. A BLAST-NEM cell population is derived by culturing a population of BLAST cells from about 3 day to about 10 days, preferably for about 6 days.

In accordance with the present invention, other culture conditions (i.e., in addition to time and medium) are also important in obtaining a BLAST-NEM cell population of the present invention from a population of BLAST cells. During culturing, variables such as cell density, temperature and CO₂ levels can be controlled to maximize the development of populations of BLAST-NEM cells. For example, it appears that the density of cells in a BLAST cell culture can affect the development of a BLAST-NEM cell population. The optimum cell

density for the growth of an BLAST-NEM cell population is from about 5×10^4 BLAST cells per ml to about 7.5×10^5 BLAST cells per ml, more preferably from about 1×10^5 BLAST cells per ml to about 6×10^5 BLAST cells per ml, and even more preferably from about 2.5×10^5 BLAST cells per ml to about 5×10^5 BLAST cells per ml. The optimum temperature for the development of an BLAST-NEM cell population is from about 35°C to about 39°C, preferably from about 36°C to 38°C, with a temperature of 37°C being even more preferred. The optimum CO₂ levels in the culturing environment for the development of BLAST-NEM cell populations is from about 3% CO₂ to about 10% CO₂, more preferably from about 4% CO₂ to about 6% CO₂, and even more preferably about 5% CO₂.

In a preferred embodiment, a BLAST-NEM cell population of the present invention is derived by culturing an individual BLAST cell colony in a medium including IMDM, with about 10% PP-FBS, 1% methyl cellulose, and a mixture of growth factors including IL-1, IL-3, IL-6, IL-11, C-kit ligand and EPO for about 6 days at about 37°C, in an about 5% CO₂-containing environment to obtain a population of BLAST-NEM cells.

The embryonic cell populations of the present invention can be used in the isolation and evaluation of compounds associated with the differentiation of embryonic cells. Thus, another aspect of the present invention is a method to identify an embryonic cell compound which, as used herein, is

a compound expressed during the development of a population of BLAST cells of the present invention from an ES cell population (i.e., including the stage of EB cell development). The method comprises characterizing the cellular composition of at least one cell contained in a population of cells including an ES cell population, a pluripotent EB cell population of the present invention, an embryonic blast cell population and intermediates thereof (i.e., cells of stages between ES and EB cell populations, or between EB and BLAST cell populations), to identify a compound expressed during the development of a population of pluripotent BLAST cells. As used herein, a cellular composition refers a composition containing components of a cell. Preferred cellular compositions of the present invention include nucleic acids, proteins, lipids (including membranes) and/or carbohydrates, with proteins, DNA molecules and RNA molecules being more preferred.

The present invention includes a variety of methods to identify an embryonic cell compound using an embryonic cell population of the present invention. In one embodiment, an embryonic cell compound of the present invention is identified by direct hybridization studies, comprising hybridizing a nucleic acid molecule probe (which can be DNA, RNA or modified forms thereof) to a composition of nucleic acid molecules isolated from an embryonic cell population of the present

invention. Such a method is useful for identifying the expression of compounds in an embryonic cell population. For example, a nucleic acid molecule encoding a protein can be hybridized under suitable conditions known to those of skill
5 on the art (see, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Labs Press, 1989) to an RNA composition isolated from an embryonic cell population of the present invention, or to a cDNA product thereof. Preferred nucleic acid molecules for use in a direct
10 hybridization study of the present invention include nucleic acid molecules that encode marker proteins including, but not limited to, endothelial cell proteins, lymphoid cell proteins, epithelial proteins, mature hematopoietic cell proteins, and/or hematopoietic stem cell proteins. As used herein, a
15 marker protein is a protein typically found in certain cell types and, as such, can suggest identification of such cell type. Particularly preferred nucleic acid molecules for use in a direct hybridization study of the present invention include nucleic acid molecules that encode proteins including
20 stem cell leukemia protein, GATA-1, GATA-2, C-Myb, C-kit ligand, C-fms, Flk-1, beta-globin, betaH1-globin, brachyury, VLA-4 and LFA-1. An embryonic cell-derived nucleic acid composition useful for such direct hybridization studies can include genomic DNA, RNA or cDNA of such RNA.

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In another embodiment, an embryonic cell compound of the present invention is identified by selective nucleic acid hybridization techniques well known to those of skill in the art. Such subtractive hybridization techniques are particularly useful for identifying novel embryonic cell compounds and for identifying compounds expressed in a given cell type. Subtractive hybridization techniques of the present invention can be performed by, for example: (1) hybridizing nucleic acid molecules isolated or derived from an embryonic cell population of the present invention to nucleic acid molecules isolated or derived from a non-embryonic cell population; or (2) hybridizing nucleic acid molecules isolated or derived from a first embryonic cell population of the present invention to nucleic acid molecules isolated or derived from a second embryonic cell population of the present invention. For example, nucleic acid molecules isolated from an EB cell population of the present invention can be subtracted from nucleic acid molecules isolated or derived from a BLAST cell population of the present invention or vice versa.

In yet another embodiment, an embryonic cell compound of the present invention is identified by nucleotide sequencing of DNA isolated from an embryonic cell population of the present invention. In order to identify compounds expressed in certain cell types, cDNA copies of poly A+ RNA is

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preferably analyzed. Identification of embryonic cell compounds can be achieved by comparing the DNA sequence information encoding such compounds derived from the embryonic cell population with sequences of known molecules. Such DNA sequencing studies are particularly useful for identifying novel embryonic cell compounds. DNA sequencing studies can be performed using techniques standard in the art (see, for example, Sambrook et al., *ibid.*).

In yet another embodiment, an embryonic cell compound of the present invention is identified by selective binding of proteins isolated from an embryonic cell population of the present invention to antibodies specific for known cellular proteins to determine the presence of such cellular proteins in the embryonic cell population. Such antibody binding studies are particularly useful for identifying the expression of known compounds by embryonic cell populations of the present invention. Antibody binding studies of the present invention can be performed using techniques standard in the art, such as by immunoblot assays, immunoprecipitation assays, enzyme immunoassays (e.g., ELISA), radioimmunoassays, immunofluorescent antibody assays and immunoelectron microscopy; see, for example, Sambrook et al., *ibid.*

In yet another embodiment, an embryonic cell compound of the present invention is identified by cell culture assays that indicate cell survival and cell proliferation. Such cell

culture assays are particularly useful for identifying both novel and known growth factors that are secreted by an embryonic cell population of the present invention. A cell culture assay of the present invention can be performed by:

- 5 (1) recovering supernatant from a culture of a dense embryonic cell population of the present invention; (2) contacting the supernatant with a sparse population of the embryonic cell population; and (3) determining if the supernatant is able to promote the survival and/or proliferation of said embryonic
- 10 cell population by observing the health of said cell population. Such cell culture assays can be performed using the cell culturing techniques disclosed in detail herein. A preferred dense population of cells includes any cell density used to culture an embryonic cell population as disclosed
- 15 herein. A preferred sparse population of an embryonic cell population of the present invention includes a cell density of from about 5×10^3 to about 2×10^5 cells per ml.

In yet another embodiment, an embryonic cell compound of the present invention involved in signal transduction in an

20 embryonic cell is identified using kinase assays that are standard in the art. Such kinase assays are particularly useful for identifying known signal transduction proteins in an embryonic cell population of the present invention.

In yet another embodiment, an embryonic cell compound of

25 the present invention is identified by protein:protein binding

studies other than antibody binding studies. In particular, embryonic cell compounds are identified by determining ligand:receptor interactions. For example, an embryonic cell population of the present invention can be contacted with a known ligand to determine if the cell population contains cells having the receptor to which the ligand can bind. Such protein:protein binding studies can be performed using techniques known to those of skill in the art.

According to the present invention, an embryonic cell compound can be a compound that has been previously identified, or not previously identified, from a cell or culture medium of a cell other than a population of cells of the present invention. For example, an embryonic cell compound of the present invention can include a growth factor that is also produced by a more mature fetal or adult cell of an animal.

An embryonic cell compound of the present invention can be a compound that is capable of having a biological effect on a cell. For example, preferred embryonic cell compounds are capable of maintaining the survival of a cell, inducing the propagation of a cell and/or stimulating the differentiation of a cell. Preferred embryonic compounds of the present invention include a compound that can be used as a marker for a population of embryonic cells. In particular, an embryonic cell marker of the present invention can be cell surface

markers, secreted molecules, cytoplasmic signal transduction molecules, transcription factors and other DNA or RNA binding proteins. As used herein, a cell surface marker refers to any compound on the surface of a cell that is detectable by techniques such as antibody binding studies, gel electrophoresis and various chromatography techniques known to those of skill in the art. A cell surface marker can include cell surface receptors, adhesion proteins, cell surface carbohydrate moieties, membrane-bound ligands and other molecules involved in cell to cell communication. A secreted molecule refers to any molecule produced and secreted by a cell into an extracellular environment and includes growth factors and other ligands. A cytoplasmic signal transduction molecule refers to a molecule that is able to regulate an intracellular chemical reaction that enables a cell to modify its biological functions based on signals in the environment, either outside or inside the cell. Signal transduction molecules can include enzymes, such as kinases, phosphatases and phospholipases. Preferred embryonic cell compounds of the present invention include a cell surface receptor, a cell surface molecule, a cytoplasmic signal transduction protein, a transcription factor, a growth factor, and DNA or RNA binding proteins.

Identification of known and novel (i.e., newly identified) compounds in an embryonic cell population of the

present invention is particularly useful for defining markers useful for the identification and/or isolation of comparable populations of cells from non-embryonic populations of cells. A particularly preferred non-embryonic cell population to look for cells having embryonic markers includes non-embryonic cell populations, including bone marrow (e.g., fetal, infant, adolescent and adult bone marrow). The presence of an embryonic cell marker of the present invention on a non-embryonic cell can indicate that the non-embryonic cell is pluripotent. Preferred embryonic cell population markers to identify comparable non-embryonic cell populations include lineage-specific markers, such as hematopoietic precursor markers, in particular pre-hematopoietic mesoderm markers. In a preferred embodiment, a population of adult bone marrow cells is screened for the presence of a cell surface marker found on the surface of an EB cell population and/or a BLAST cell population of the present invention.

On embodiment of the present invention is a formulation that contains one or more isolated embryonic cell compounds of the present invention that can be used for therapeutic or experimental use. According to the present invention, an isolated embryonic cell compound is a compound that has been removed from its natural milieu. An isolated embryonic cell compound can, for example, be obtained from its natural source, be produced using recombinant DNA technology, or be

synthesized chemically. Preferred embryonic cell compounds of the present invention, including homologues thereof, are capable of regulating embryonic development. A preferred embryonic cell compound homologue includes at least one epitope capable of effecting differentiation of an ES cell population. The ability of an embryonic cell compound homologue to effect differentiation of an ES cell population can be tested using techniques disclosed herein. A preferred formulation of the present invention includes at least one protein secreted by a cell contained in an EB cell population of the present invention and/or a BLAST cell population of the present invention. Preferably, a formulation of the present invention comprises a culture supernatant obtained by culturing an EB cell and/or BLAST cell population of the present invention.

Another aspect of the present invention comprises an antibody capable of binding to an embryonic cell compound of the present invention. Binding can be measured using a variety of methods known to those skilled in the art including immunoblot assays, immunoprecipitation assays, enzyme immunoassays (e.g., ELISA), radioimmunoassays, immunofluorescent antibody assays and immunoelectron microscopy; see, for example, Sambrook et al., *ibid.* Antibodies of the present invention can be either polyclonal or monoclonal antibodies. Antibodies of the present invention

include functional equivalents such as antibody fragments and genetically-engineered antibodies, including single chain antibodies, that are capable of selectively binding to at least one of the epitopes of the protein or mimotope used to obtain the antibodies. Preferred antibodies are raised in response to surface marker proteins of an embryonic cell population of the present invention, in particular, surface cell receptors. Antibodies of the present invention can be produced using methods standard in the art. Antibodies of the present invention are particularly useful for identifying and isolating populations of cells having such surface markers, in particular, populations of embryonic cells from different species of animals and/or cells with similar markers from adult bone marrow. Thus, particularly preferred antibodies of the present invention include antibodies that are capable of binding to cellular markers that delineate between different embryonic cell populations of the present invention.

Another aspect of the present invention is a therapeutic composition that comprises an embryonic cell population of the present invention, which is capable of serving as a population of cells that act as progenitors of various lineages. The therapeutic composition can be particularly useful to repopulate one or more lineages in an animal. As used herein, the term repopulate refers to a cell population that can be administered to an animal to restore a lineage of cells. A

therapeutic composition of the present invention can be useful for the treatment of disease, such as anemia, leukemia, breast cancer and AIDS. A therapeutic composition of the present invention can be particularly useful for enhancing populations of adult bone marrow cells used in transplantation procedures. A preferred therapeutic composition of the present invention includes a population of EB cells of the present invention and/or a population of BLAST cells of the present invention. A more preferred therapeutic composition of the present invention includes a population of EB cells of the present invention and/or a population of BLAST cells of the present invention derived from an ES cell population derived from a mammalian embryo. An even more preferred therapeutic composition of the present invention includes a population of EB cells of the present invention and/or a population of BLAST cells of the present invention derived from an ES cell population derived from a human embryo.

Therapeutic compositions of the present invention can be administered to any animal, preferably to mammals, and more preferably to humans. Therapeutic compositions of the present invention can be formulated in an excipient that the animal to be treated can tolerate and that maintains the integrity of the embryonic cell population. Examples of such excipients include aqueous physiologically balanced salt solutions.

Excipients can also contain minor amounts of additives, such as substances that enhance isotonicity and chemical stability.

Another aspect of the present invention is the use of embryonic cell populations of the present invention for the treatment of genetic diseases. Genetic diseases associated with various lineages can be treated by genetic modification of autologous or allogenic populations of embryonic cells of the present invention. For example, diseases such as beta-thalassemia, sickle cell anemia, adenosine deaminase deficiency and other genetic diseases related to a deficiency or malfunction of a cell of hematopoietic lineage, can be corrected by introduction of a wild type gene into the embryonic cell population. Diseases other than those associated with hematopoietic cells can be treated, where the disease is related to the lack of a particular secreted product, such as a hormone, enzyme, growth factor and the like. Specific promoters can be employed based upon identification of transcription factors of an embryonic cell population as described herein. Thus, inducible production of a desired product encoded by transformed genes can be achieved. Methods for transformation and expression of genes in an embryonic cell population of the present invention are standard to those in the art (see, for example, Sambrook et al., *ibid.*).

In accordance with the present invention, a nucleic acid molecule can be transformed into an embryonic cell population of the present invention to inhibit particular gene products, thereby inhibiting susceptibility to a disease. For example, 5 an embryonic cell population of the present invention can be transformed with a ribozyme, or a nucleic acid molecule that is capable of homologous recombination or antisense expression. For example, a BLAST cell population of the present invention can be transformed with a gene that disrupts 10 the expression of a specific T cell receptor gene and administered to an animal. Subsequent differentiation of the BLAST cell population to a T cell population results in the production of a population of T cell receptor negative T cells. Such a method could be effective for preventing or 15 treating autoimmune disease which involve autoreactive T cell activity. Similarly, an embryonic cell population of the present invention can be modified to introduce an antisense sequence or ribozyme to prevent proliferation of any pathogen that uses proteins of an animal cell to proliferate (e.g., 20 viruses) in an embryonic cell population, or in progenitors or progeny thereof.

The following experimental results are provided for purposes of illustration and are not intended to limit the scope of the invention.

EXAMPLES

Example 1

This example describes the production of a population of embryoid body cells from an embryonic stem cell population.

5 The CCE ES cell line, originally derived from a 129/Sv/Ev strain of mouse, were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 15% fetal calf serum (FCS), 1.5×10^{-4} monothioglycerol (MTG), and Leukemia Inhibiting Factor (LIF). The ES cells were passaged every 2-3 days at a
10 dilution of approximately 1:15. Two days before the initiation of the differentiation cultures, the undifferentiated ES cells were passaged into Iscove's modified Dulbecco's medium (IMDM) supplemented with the above components. To induce differentiation into an EB population
15 of cells, the ES cells were trypsinized, washed, and counted using techniques standard in the art. The freshly dissociated ES cells were then cultured in IMDM containing 15% platelet-derived fetal bovine serum (PDS; obtained from Antech, TEXAS; also referred to herein as platelet-poor fetal bovine serum,
20 PP-FBS), 4.5×10^{-4} M MTG, transferrin (300 μ g), glutamine (2 mM). The ES cells were plated in a final volume of 10 ml at a concentration of about 3000 to about 4500 cells per ml of medium in 150 mm bacterial grade dishes. The ES cell population was then cultured in a humidified environment of 5%
25 CO₂, at a temperature of 37°C. At an appropriate time after

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the initiation of differentiation (i.e., plating of cells in PP-FBS), EB cell populations were harvested using standard techniques. The cells were then centrifuged at x1000 rpm to pellet the cells and the supernatant was removed. The EB
5 cells were resuspended in 3 ml trypsin for 1 to 3 minutes at 37°C. An equal volume of IMDM containing 5 to 10% FCS was added to the trypsinized cells. A single cell suspension of EB cells was achieved by passing the EB cells 3 to 4 times through a 3 ml or 5 ml syringe with a 20 gauge needle.

10 The EB cell colonies were viewed under a Leitz inverted light microscope and were found to generally consist of groups of tightly packed cells, in which individual cells were not easily detectable. A representative microscopic view of an EB cell colony is shown in Fig. 2, cell colony A. The EB cell
15 colony consisted of a tightly packed group of cells, in which single cells were essentially not discernable.

Example 2

This example describes the labelling of a population of EB cells using antibodies specific for known cell surface
20 molecules.

EB cells derived from ES cells that had been incubated for about 4 days (Day 4 EB) according to the method described in Example 1 were labelled for fluorescence activated cell
25 sorter analysis using a panel of antibodies against a number of different surface markers. These included antibodies

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specific for AA4.1, Sca-1, C-kit receptor, H-2b, VLA-4, CD44, CD45 and Thy 1. In addition, a population of CCE ES cells, similar to those cells used to derive the EB cell population, were stained under the same conditions as the EB cell population as a control sample. The results are shown in Fig. 3A and 3B. Referring to Fig. 3A and 3B, the Day 4 EB cells (d4 EB) stained with low amounts of anti-Sca-1, anti-C-kit receptor and anti-H-2b antibodies, and essentially no anti-Thy 1, anti-VLA-4, anti-CD44 and anti-CD45 antibodies, thereby indicating low or no expression of the corresponding surface marker protein. In addition, comparing the staining pattern of the EB cells to the control ES cells, the EB cells express slightly higher levels of C-kit receptor and H-2b protein but slightly less Sca-1 protein. Thus, the results show that expression of certain surface antigens change over a period of 4 days during development of ES cells to EB cells.

Example 3

This example describes the production of a population of embryonic blast cells from an embryoid body cell population. EB cells were generated as described in Example 1. A series of cultures were prepared by plating about 2×10^5 to about 5×10^5 EB cells, derived according to the method described in Example 1. The EB cells were cultured in 1% methyl cellulose made in IMDM containing 10% PP-FBS, transferrin (300 $\mu\text{g/ml}$), glutamine (2 mM), and either a

mixture of IL-1 (1000 units/ml), IL-6 (5 ng/ml), IL-11 (25 ng/ml), C-kit ligand (100 ng/ml), or C-kit ligand (100 ng/ml), VEGF (5 ng/ml) and EPO (2 units/ml), or C-kit ligand (100 ng/ml) alone. The EB cells were cultured in a final volume of 1 ml in a 35 mm bacterial grade dishes in a humidified environment of 5% CO₂ at 37°C. Individual EB cell cultures were incubated for 3 days, 3.5 days, 4 days and 4.5 days. BLAST cell colonies identified using a Leitz inverted light microscope based on morphology. A representative BLAST cell colony is shown in Fig. 2, cell colony B. The BLAST cell colony consisted of a clumped, but not tightly packed, group of cells in which individual cells could be discerned in the colony.

The formation of BLAST cell colonies at each time point was scored by eye and the kinetics of BLAST cell development are shown in Fig. 5. The results indicate that BLAST cell (Blast) development was detected within 3 days of initiation of EB cell (EBs) incubation under the above culture conditions, increasing slightly in number by day 3.5 and then decreasing to low levels by day 4.5. As the number of BLAST cells increased, the number of EB cells decreased. In addition, as the number of BLAST cells decreased, the number of erythroid cells (Ery), which were scored by red color, appeared at day 4 and continued to increase through day 4.5.

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Example 4

This example describes the labelling of a population of BLAST cells using antibodies specific for known cell surface molecules.

5 EB cells were generated as described in Example 1. BLAST cells were generated from EB cells that had been incubated for about 6 days (Day 6 BLAST) according to the method described in Example 3. The BLAST cells were labelled for fluorescence activated cell sorter analysis using a panel of antibodies
10 against a number of different surface markers. These included antibodies specific for AA4.1, Sca-1, C-kit receptor, H-2b, VLA-4, CD44, CD45 and Thy 1. ES cells and Day 4 EB cells (described in Example 2) were also stained as control samples. The results are shown in Fig. 3. Referring to Fig. 3, the Day
15 6 BLAST cells (d6 Blasts) stained with anti-CD44, anti-C-kit receptor, anti-Sca-1 and anti-VLA-4 antibodies, but not with anti-H-2b, anti-Thy 1 or anti-CD45 antibodies. Thus, indicating that Day 6 BLASTS express substantial amounts of CD44, C-kit receptor, Sca-1 and VLA-4 protein, and essentially
20 no Class I H-2^b, Thy 1 and CD45 protein. Comparing the staining pattern of the Day 6 BLASTS with the EB cell and ES cells, the results show that expression of C-kit receptor, H-2b, VLA-4 and CD44 increases during the 6 days of development of EB cells to BLAST cells.

Example 5

This example describes a myeloid assay to test for hematopoietic precursors in BLAST cell populations.

A schematic representation of the myeloid assay used to
5 identify secondary hematopoietic colony formation from BLAST
cells is shown in Fig. 6. Two different culture experiments
were prepared as follows. In a first experiment, EB cells
were generated as described in Example 1 by culturing ES cells
for 4 days. Individual BLAST cell colonies were generated by
10 incubating the EB cell population for 3, 4, 5 and 6 days
according to the method described in Example 3. In a second
experiment, EB cells were generated as described in Example 1
by culturing ES cells for 3, 3.5, 4 and 4.5 days. Individual
BLAST cell colonies were generated by incubating each EB cell
15 population for 6 days according to the method described in
Example 3. In each experiment, individual BLAST cell colonies
were picked, dispersed in 100 μ l IMDM containing 5% FCS, and
transferred to a 1% methyl cellulose culture containing IMDM,
10% PP-FBS, 300 μ g transferrin and a cocktail of growth
20 factors including IL-1 (1000 units/ml), IL-3 (100 units/ml),
IL-6 (5 ng/ml), IL-11 (25 ng/ml), C-kit ligand (100 ng/ml) and
EPO (2 units/ml). The cultures were then incubated for
varying amounts of time in a humidified 5% CO₂, at a
temperature of 37°C.

In the first experiment, developing hematopoietic colonies were scored 7 days after initiation of the culture. In the second experiment, developing hematopoietic colonies were scored 3, 4, 5 and 6 days after initiation of the culture. The growth of hematopoietic colonies were scored based on colony morphology when the colonies were viewed under an inverted Leitz light microscope, and based on cellular staining patterns. Erythrocytes, macrophages, neutrophils and mast cells were identified in all cultures having hematopoietic cell development.

The results from the first experiment indicated that as many as 70% BLAST cells gave rise to hematopoietic colonies (see Fig. 7A). Kinetic analysis revealed that BLAST cell colonies incubated for 3 days gave rise to fewer hematopoietic colonies than BLAST cell colonies incubated for 6 days (see Fig. 7B). Thus, indicating that a maturation process occurred within the BLAST cell population between days 3 and 6.

In the second experiment, the percent of total BLAST cell colonies that generated a secondary hematopoietic colony, including multi- or uni-lineage colonies at each time point was determined. Multi-lineage refers to colonies that contain erythroid plus two other lineages. In addition, the percent of total BLAST cell colonies that generated that generated only multi-lineage colonies at each time point was determined. The results are shown in Fig. 8. Referring to the percent of

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cells that replate to multi-lineages (black bars), the results indicate that BLAST cell colonies generated from day 3 EB cells contain more immature hematopoietic cells than those derived from day 4.5 EB cells based on the generation of multi-lineage colonies.

Morphological analysis of the secondary hematopoietic populations arising from BLAST cells indicated the presence of primitive erythroid (Ery^p), definitive erythroid (Ery^d) and multiple myeloid cells in a single replated culture. The results are summarized in Table 1.

Table 1. Incidence of Primitive and Definitive Erythroid Colonies from Individually Replated Blast Colonies

Colony Type	Ery ^p	Ery ^d (± myeloid)	Ery ^p + Ery ^d (+ myeloid)
Blast Colonies	8	140	10

The presence of both primitive and definitive erythroid cells in a BLAST cell colony indicates that the BLAST cell population that gave rise to the erythroid cell populations had the potential to generate all hematopoietic populations and represents one of the earliest hematopoietic cells to develop, equivalent to a pre-yolk sac cell (see Fig. 9, cell A). A certain number of BLAST cells generated predominantly primitive erythroid cells, while others generated definitive erythroid cells and cells of the various myeloid lineages. These latter two patterns of replating indicate that some of the BLAST colonies are committed to primitive erythropoiesis

(analogous to the cells that expand in the yolk sac) (Fig. 9, cell B); while others have lost the capacity to generate this early lineage, but can generate all other populations (equivalent to the cells that ultimately seed the fetal liver) (Fig. 9, cell C).

Example 6

This example describes the influence of individual growth factors on hematopoietic colony formation from BLAST cell colonies.

BLAST cell populations were generated from Day 3.5 EB cell populations according to the method described in Example 3 were then incubated in the presence of specific growth factors (shown in Fig. 10). A control sample was prepared that contained no factors (- Factor). Twenty-five individual BLAST cell colonies were picked and replated into a myeloid assay as described in Example 5. The results shown in Fig. 10A indicate that EPO was the least effective growth factor in stimulating the formation of BLAST cell colonies capable of developing into any type of secondary hematopoietic colony. The results shown in Fig. 10B indicate that IL-6 and EPO were the least effective growth factors in stimulating the formation of BLAST cell colonies capable of developing into multi-lineage secondary hematopoietic colonies. C-kit ligand and a mixture of C-kit ligand, EPO and VEGF were most effective in stimulating the formation of BLAST cell colonies

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capable of developing into any type of hematopoietic colony including colonies having multi-lineage colonies.

Example 7

This example describes the formation of a T cell
5 population from an ES cell population using a mixture of IL-7, IGF-1 and C-kit ligand growth factors.

EB cells were generated from ES cells according to the method described in Example 1. BLAST cell colonies were generated from the EB cells according to the method described
10 in Example 3. Individual BLAST cell colonies were picked, dispersed in IMDM containing 5% FCS, and transferred to a 1% methyl cellulose culture containing IMDM, 10% PP-FBS, 300 μ g transferrin and a cocktail of growth factors including IL-7, IGF-1 and C-kit ligand. The cultures were then incubated for
15 about 6 days in a humidified 5% CO₂, at a temperature of 37°C.

Thymi were obtained from pregnant (15 days gestation) outbred Swiss Webster mice (purchased from Taconic) which were found to express the Thy 1.1 allele. The thymi were then irradiated at a dose of 3000Gy to deplete endogenous cells.
20 Pools of 20 BLAST cell colonies were seeded into each thymic lobe in a hanging drop culture in a terrasacki well and incubated for 48 hours. Following the 48 hour hanging-drop culture, the thymi were harvested into IMDM with 10% FCS and transferred to sterile 45 micron filters (Gelman) which were
25 placed on gelfoam sponges (Upjohn) for 3 weeks at the air

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medium interface. The thymi were then removed from the filter and dissociated by treatment with .25% collagenase (Sigma, St. Louis, MO), 10 μ g/ml DNase in phosphate buffered saline (PBS) and digested for 1 hour at 37°C. Following digestion, the
5 thymi were dispersed by passaging the cells through a 3 ml syringe attached to a 20 gauge needle.

The resulting single cell suspension was stained for host Thy 1.1, donor Thy 1.2 and T cell receptor expression by the following method. About 4×10^4 cells were separately
10 incubated with FITC labelled anti-Thy 1.2 antibody (1:1000; Pharminogen, San Diego, CA), FITC labelled anti-Thy 1.1 antibody (1:1000; Pharminogen), phycoerythrin (PE) labelled anti- $\alpha\beta$ T cell receptor antibody (1:100; Pharminogen), and PE labelled anti- $\gamma\delta$ T cell receptor antibody (1:100; Pharminogen)
15 for 20 minutes, on ice. The cells were washed and analyzed on a FACSCAN (Becton Dickenson).

Referring to Fig. 11, donor Thy 1.2 positive cells expressing both $\alpha\beta$ and $\gamma\delta$ T cell receptor were detected indicating that a mature T cell population was derived from
20 the donor BLAST cell colonies that had been treated with IL-7, IGF-1 and C-kit ligand.

Example 8

This example describes the formation of a T cell population from an ES cell population using varying
25 combinations of growth factors.

BLAST cell colonies were generated as described in Example 7. Individual BLAST cell colonies were then incubated with different combinations of growth factors including C-kit ligand alone, a mixture of C-kit ligand and IL-7, a mixture of C-kit ligand, IL-7 and IGF-1 and a mixture of C-kit ligand, VEGF and EPO. Using the method described in Example 7, the BLAST cell colonies were incubated, T cell populations were derived, and the resulting T cell populations were stained with anti-Thy 1.2 and $\alpha\beta$ T cell receptor antibodies.

Referring to Fig. 12, incubation of BLAST cell colonies with the combination of C-kit ligand, IL-7 and IGF-1 growth factors (BLAST KL/IGF-1/IL-7) produced the most $\alpha\beta$ T cell receptor positive T cells. The presence of the other combinations of factors, however, also produced some $\alpha\beta$ T cell receptor positive T cells. A positive control sample of bone marrow stromal cells BLAST cell colonies (BM) produced similar numbers of $\alpha\beta$ T cell receptor positive T cells as the KL/IGF-1/IL-7 treated BLAST cell colonies. Thus, optimal conditions for production of T cell populations include incubation of BLAST cell colonies with a mixture of C-kit ligand, IL-7 and IGF-1 growth factors.

Example 9

This example describes the formation of a B cell population from an ES cell population.

EB cell colonies were generated from ES cells using the method of Example 1. BLAST cell colonies were derived from EB colonies using the method of Example 3. Individual BLAST cell colonies were picked from methyl cellulose culture and transferred to confluent monolayers of S17 bone marrow stromal cells that had been irradiated at a dose of 3000Gy. Cultures were grown for 4 weeks in IMDM with 5% FCS and C-kit ligand. cDNA samples were prepared from the C-kit ligand treated cells by dispersing the cells using trypsin, lysing the cells and preparing cDNA using reverse transcriptase (BRL Gibco) according to methods standard in the art. cDNA samples were then amplified by PCR for VDJ immunoglobulin rearrangement using a 5' VH7183 primer (5'-TGGTGGAGTCTGGGGGAGGCTTA-3') and a 3' JH4 primer (5'-GGCTCCCTCAGGGACAAATATCCA-3') using the following PCR profile: 94°C 1 minute, 72°C 2 minutes for 29 cycles; 94°C 1 minute, 72°C 10 minutes for one cycle. PCR products were subjected to southern blotting and hybridized with a probe complimentary to a sequence common to immunoglobulin J regions.

The results indicate that treatment of BLAST cells with C-kit ligand in addition to exposure to bone marrow stromal cells results in the production of a B cell population containing cells having rearranged immunoglobulin VDJ genes.

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Example 10

This example describes the development mixed populations of erythroid and endothelial cells from EB cell populations.

A. Mixed Endothelial and Erythroid Population Development

5 Approximately 2×10^5 EB cells derived from CCE ES cells that were cultured for about 4 days according to the method described in Example 1 were dissociated with trypsin and re-plated and cultured in 1% methyl cellulose made in IMDM containing 10% PP-FBS, transferrin (300 μ g/ml), glutamine (2
10 mM), VEGF (5 ng/ml) and EPO (2 units/ml). The EB cells were cultured in a final volume of 1 ml in a 35 mm bacterial grade dishes in a humidified environment of 5% CO₂ at 37°C. The EB cells were cultured for about 7 days.

The resulting "mixed" cell population was viewed under an
15 inverted Leitz microscope. Under the above culture conditions, 3 different morphological types of cell colonies arose and representative cells are shown in Fig. 4. A first cell type, indicated as cell A in Fig. 4, consisted of an erythroid cell having the typical characteristics of a
20 distinct compact cluster of small cells having red color. A second cell type, indicated as cell B in Fig. 4, consisted of a spherical cell having a larger size than an erythroid cell, such as cell A. A third cell type, indicated as cell C in Fig. 4, consisted of a spherical cell having a similar size as

an erythroid cell but having a single long process extending from the cell.

B. Kinetics of Mixed Erythroid and Endothelial Population Development

5 Varying EB cell populations were generated using the method described above in section A by incubating ES cells for 4, 5, 6 and 7 days. Various types of colonies were then scored about 7 days following plating based on the 3 different morphologies described in Section A and cultures containing
10 erythroid (ERY) and non-erythroid (NON-ERY) cells were scored as "mixed" (MIX) populations. Referring to Fig. 13, the results indicate that the number of mix colonies decreases with increasing age of EB cells and are almost undetectable by day 7. Meanwhile, erythroid colonies increased in number
15 between days 4 and 6 of differentiation.

C. Influence of Specific Growth Factors on Mixed Erythroid and Endothelial Population Development

An EB cell population was generated using the method described above in section A by incubating ES cells for 4
20 days. In a first experiment, the effect of specific growth factors on the development of mixed endothelial and erythroid cell populations was tested. Separate populations of Day 4 EB cells were plated in the presence of EPO (2 units/ml) alone, VEGF (5 ng/ml) alone, a mixture of EPO and VEGF, or no factor.
25 The cultures were then scored for erythroid (ERY), non-

erythroid (NON-ERY) cells and mixed (MIX) cell populations as described above. Referring to Fig. 14, the results indicate that the combination of EPO and VEGF growth factors induced the best EB cell differentiation, in particular to mixed cell populations.

In a second experiment, the concentration of specific growth factors on the development of mixed endothelial and erythroid cell populations was tested. Separate populations of Day 4 EB cells were plated in the presence of 0 nanograms (ng) per ml of EPO + VEGF (E/V/0), 5 ng/ml EPO + VEGF (E/V/5), 10 ng/ml EPO + VEGF (E/V/10), 15 ng/ml EPO + VEGF (E/V/15), 30 ng/ml EPO + VEGF (E/V/30), and no factor (-F). The cultures were then scored for erythroid (ERY), non-erythroid (NON-ERY) cells and mixed (MIX) cell populations as described above. Referring to Fig. 15, the results indicate that 5 ng/ml of the combination of EPO and VEGF growth factors induced the best EB cell differentiation, in particular to mixed cell populations.

D. von Willebrand Factor and Acetylated-LDL Staining of Mixed Cell Populations

A mixed population of cells derived from the method described in Section A was stained with the endothelial cell specific marker von Willebrand factor (vWF) and diI-Acetylated-low density lipoproteins (DiI-Ac-LDL). The mixed colonies were picked from methyl cellulose culture and allowed to adhere overnight to cover slips that were coated with poly-

L-lysine or gelatin. Cells were stained for vWF by the following method. Cells attached to cover slips were fixed for 10 minutes in a solution containing 3% paraformaldehyde and 3% sucrose in PBS. After washing 2 to 3 times, the cells
5 were permeabilized with 0.2% Triton X-100 in PBS. A solution of normal mouse serum and human immunoglobulins was used to block non-specific binding. Subsequently, the cells were incubated with a rabbit anti-human vWF antibody for 1 hour and washed 5 times, 5 minutes each wash. The cover slips were
10 then incubated with horse radish peroxidase labelled goat anti-rabbit antibody (obtained from Fisher) for 1 hour and washed for 5 times, 5 minutes each wash. The labelled cells were then incubated for 15 minutes in a solution containing 0.5 mg/ml of diaminobenzidine, 3 mg/ml nickel sulfate, 0.003%
15 H_2O_2 , and 100 mM Tris (pH 7.5).

The vWF stained cells were analyzed by eye. The results indicated that two of the three cell types identified in the mixed population (described above in Section A) were labelled with vWF. A representative field of stained cells is shown in
20 Fig. 16. Referring to Fig. 16A, the cells described in Section A that had long processes stained with vWF. Referring to Fig. 16B, the cells described in Section A that were larger than the red erythroid cells also stained with vWF. The red erythroid-like cells identified in section A did not stain
25 with vWF. Referring to Fig. 16C, a control population of

macrophage cells also failed to stain with vWF. These results indicate that the non-erythroid cells in the mixed population having long processes or having a larger size than the erythroid cells stain with an endothelial cell specific
5 marker.

Cells were tested for LDL uptake by the following method. Ten $\mu\text{g/ml}$ DiI-Ac-LDL was added to the medium of cultures of mixed cell populations and incubated for 4 hours at 37°C . Following incubation, the cells were washed 3 times and fixed
10 with a solution of 3% paraformaldehyde and 3% sucrose in PBS. Following fixation, the cells were mounted on glass slides using 90% glycerol and 10% PBS and analyzed by eye. The results indicated that the non-erythroid cells in the mixed population having long processes or having a larger size than
15 the erythroid cells took up the DiI-Ac-LDL, an endothelial cell specific stain.

Taken together, the observation that the non-erythroid cells generated in the presence of VEGF stained with both vWF and DiI-Ac-LDL indicates that the non-erythroid cells
20 represent endothelial cells and therefore the mixed colonies appear to contain both hematopoietic and endothelial cells.

Example 11

This example demonstrates that erythroid and endothelial cells in a mixed population arose from a common precursor
25 cell.

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EB cell colonies were generated using the method described in Example 1. To determine if the differentiation of EB cells in the presence of VEGF and EPO was significantly reduced in GATA-2⁻ EB cells, varying numbers of GATA-2⁺ EB cells (see Fig. 17) were plated into Methyl cellulose cultures containing VEGF, EPO and 2×10^5 GATA-2⁻ EB cells (as feeder cells; kindly provided by Dr. Stuart Orkin at Children's hospital in Boston). The cultures were then analyzed for the development of mixed colonies under an inverted Leitz microscope. It was found that when a standard number of GATA-2⁻ EB cells were added to cultures containing varying numbers of GATA-2⁺ EB cells, a linear relationship between the number of mixed colonies and the number of GATA-2⁺ EB cells was observed. When plotted in a log:log format, the slope of this relationship was not significantly different from 1, a finding which indicates that the colonies derive from a single cell (see Fig. 17).

Example 12

This example describes confirmation studies of the single cell origin of mixed colonies using a retroviral marking technique.

Retroviruses that carry unique inserts in addition to G418 resistant marker (retroviral vector LNCX based) were used to mark EB cell populations generated according to the method of Example 1. Day 4 EB cell populations were dissociated with

trypsin and incubated with retroviruses in the presence of polybene (5 µg/ml), VEGF, EPO, and IGF-1. After 5-8 hours, the cells were incubated in a 1% methyl cellulose culture with VEGF, EPO and G418 (150 µg/ml). Cells were routinely dispersed using a syringe attached to a 20 gauge needle because the cells tended to aggregate during viral infection. The resulting mixed colonies were picked into 0.1X PBS and lysed for 8 minutes at 95°C, treated with proteinase K, heat denatured for 8 minutes at 95°C, and subjected to PCR amplification. LNCX retroviral vector sequence was used for the cDNA amplification. An upstream primer (5'-CGCGGCCCCAAGCTTGTTAACATCGATGGATG-3') and a downstream primer (5'-GGCGTTACTTAAGCTAGCTTGCCAAAGGTAC-3') were used. PCR products were gel purified and subjected to further amplification. After PCR amplification, excessive primers were removed by filtration through centricon 30 filters and the DNA was concentrated by ethanol precipitation. The presence of the insert sequence at the junction of cDNA insert and LNCX retroviral vector was analyzed in 7 G418 resistant mixed colonies by sequencing using standard dideoxy sequencing methods.

The sequence analysis indicated that all of the G418 resistant mixed colonies contained the same insert sequence, thereby indicating that the mixed colonies arose from a single clone.

In summary, the results from experiments involving mixed endothelial and erythroid cell population formation indicate that there is a close association of erythroid and endothelial cell development from a common precursor. A mixed erythroid and non-erythroid cell population can be generated using the growth factors VEGF and EPO. The relationship between the number of mixed colonies generated and the number of cells plated was linear and individual mixed colonies marked with unique retroviruses revealed that these colonies mixed colonies are derived from a common precursor that gave rise to both blood and endothelial cells.

While various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur to those skilled in the art. It is to be expressly understood, however, that such modifications and adaptations are within the scope of the present invention, as set forth in the following claims:

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